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Rolling Circle Amplification, a Powerful Tool for Genetic and Functional Studies of Complete Hepatitis B Virus Genomes from Low-Level Infections and for Directly Probing Covalently Closed Circular DNA▽

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Complete characterization of the biological properties of hepatitis B virus (HBV) variants requires the generation of full-length genomes. The aim of this study was to develop new tools for the efficient full-length genome amplification of virus from samples with low viral loads. Rolling circle amplification (RCA) was used to amplify full-length HBV genomes from both sera and liver biopsy samples from chronic HBV carriers. Serum-derived relaxed circular HBV DNA could be amplified only after completion and ligation of plus-strand DNA. Covalently closed circular DNA (cccDNA) from liver biopsies could be amplified directly from as few as 13 copies, using RCA, followed by a full-length HBV PCR. Three serial liver biopsy samples were obtained from a lamivudine-resistant patient who cleared detectable serum HBV after adefovir dipivoxil was added to the lamivudine therapy and then seroconverted to anti-HBs. Only the genomes from the last biopsy specimen obtained after the emergence of lamivudine resistance contained the lamivudine resistance-associated mutations rtL180M and rtM204V ("rt" indicates reverse transcriptase domain). Defective genomes were also found in this biopsy sample. Genomes cloned from the liver biopsy specimens were transfected into HuH7 cells to study their replication competence and their susceptibility to lamivudine. RCA is a powerful tool for amplifying full-length HBV genomes and will be especially useful for the study of occult or inactive HBV infections and patients undergoing antiviral treatment. It can also be used to probe HBV cccDNA, the crucial intermediate in viral persistence and the archive of resistance mutations.

Chronic hepatitis B virus (HBV) infection is widespread, with an estimated 370 million carriers worldwide (1). Despite constraints imposed by a complex genetic architecture involving overlapping genes, the virus is highly variable, with eight known genotypes designated A to H (20).

The complete molecular and biological characterization of HBV variants requires the isolation of full-length genomes. This has been done classically by amplifying overlapping fragments from serum-derived relaxed circular genomes (RC-DNA) (4, 21). However, the discontinuous structure of RC-DNA means that the amplification of at least one of the fragments can be problematical. Increasing the number of PCRs ineluctably augments the probability of incorporating PCR errors, and further steps are necessary to construct a full-length HBV genome. Günther et al. have developed an elegant method for amplifying full-length HBV genomes in a one-step PCR (7). However, this method requires serum with viral loads of $>10^4$ copies/ml of HBV DNA. In typical chronic HBV infections, viral loads usually range from 10^6 to 10^{10} copies/ml. However, for inactive carriers (individuals who are hepatitis B surface antigen positive [$HBsAg^+$]) with minimal virus replication and for occult HBV-infected patients ($HBsAg^-$), serum HBV DNA levels are usually very much lower. Consequently, few occult HBV infections have been studied at the genomic level and then sometimes only after the occult infection has been reactivated. We therefore wanted to develop a method for the efficient and high-fidelity amplification of full-length HBV genomes from biological samples with low levels of HBV DNA. The method chosen was rolling circle amplification (RCA), also called ramification amplification (26). This method relies on the properties of the *phi29* phage DNA polymerase that possesses a strong 3′ exonuclease (proofreading) activity, that is very processive, i.e., is able to polymerize more than 70,000 nucleotides without detaching from the template, and that can displace previously elongated strands (2). On a circular template there is linear amplification (70,000 nucleotides represents more than 20 HBV genomes), and more importantly, there is branching amplification, as displaced strands reveal more binding sites for the amplification primers. The end product is a high-molecular-weight double-stranded DNA containing multiple copies of the initial target. RCA exhibits less amplification bias and greater yield, product length, and fidelity than PCR (5, 13). We have adapted this method to amplify full-length HBV genomes from the covalently closed circular DNA (cccDNA) form of the genome found in the nuclei of infected hepatocytes and from RC-DNA found in circulating virions. This method has been used here to study, as an extreme example, the residual cccDNA genomes

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TABLE 1. HBV-specific primers used for rolling circle amplification and PCR

Primer	Sequence[a]	Nucleotide position[b]
RCA1	AATCCTCACAATA*C*C	226–240
RCA2	GATGGGATGGGAA*T*A	615–601
RCA3	CCTATGGGAGTGG*G*C	637–651
RCA4	GCAACGGGGTAAA*G*G	1154–1140
RCA5	ATGCAACTTTTTC*A*C	1814–1828
RCA6	TCCAAATTCTTTA*T*A	1930–1916
RCA7	TAGAAGAAGAACT*C*C	2368–2682
RCA8	AGAATATGGTGAC*C*C	2828–2814
P1	ccggaaagcttatgctcttcT↓TTTT↑CACCTC TGCCTARTCATC	1821–1843
P2	ccggagagctcatgctcttcA↓AAA↑AGTTGC ATGGTGCTGGTG	1825–1804

[a] HBV sequences are in upper-case letters; the cloning tails are in lower-case letters. The LguI recognition sites are underlined, and arrows indicate the LguI cutting sites. * indicates phosphorothioate modifications.
[b] Nucleotides use the genotype D numbering system.

from a patient who has seroconverted to anti-HBs following successful add-on antiviral therapy.

MATERIALS AND METHODS

HBV cccDNA extraction from liver biopsy specimens. Liver biopsy specimens were collected from a chronic hepatitis B carrier and an HBV-negative patient and stored at −80°C. The chronic carrier has been followed for several years in our laboratory (17). cccDNA was extracted as described previously (24), and the copy number was quantified using quantitative PCR (23). Ten-fold serial dilutions of the cccDNA stock were carried out using material extracted from the HBV-negative liver biopsy the same way.

Isolation of DNA from serum. Nucleic acids were extracted from 150 μl of serum from a chronic HBV carrier, using a QIAamp UltraSens virus kit (Qiagen, Courtaboeuf, France). This patient has also been previously studied in our laboratory (16), and the complete sequence (of genotype C) is known.

Completion and ligation of the HBV RC-DNA plus strand. Plus-strand DNA was completed using the endogenous polymerase activity of HBV nucleocapsids before extraction. Seventy-five microliters of 3× reaction buffer was made fresh before use (21.6 μl of Tris-HCl [pH 7.6], 0.8 M; 10.8 μl of $MgCl_2$, 0.8 M;10.8 μl of NH_4Cl, 1.2 M; 10.8 μl of 2-mercaptoethanol, 3%; 10.05 μl of NP-40, 20%; 2,2 μl of each deoxynucleoside triphosphate [dNTP], 10 mM; quantity sufficient for 75 μl with sterile water), and then 150 μl of serum was added. The reaction was carried out for 12 h at 37°C in a humid chamber. The sample was then treated with 10 U of RNase-free DNase I for 10 min at 37°C, followed by extraction as described above for serum samples. For ligation, 8.5 μl of the extraction product was added to 1× ligation buffer and 1.5 U of T4 DNA ligase (Promega, Charbonnières, France) in a final volume of 10 μl. The ligation reaction was carried out for 16 h at 16°C, followed by heating at 65°C for 10 min.

PCR amplification of whole HBV genomes. Full-length HBV genomes were amplified using a one-step PCR method (7). Briefly, the method was originally based on the unusual structure of HBV RC-DNA, which has a short and A/T-rich terminal redundancy on minus-strand DNA but can also be used to amplify unit-length HBV genomes from RCA products. The sense and antisense primers are situated at the terminal redundancy and partially overlap (Table 1). In both primers, the recognition site for LguI (Sap I) is immediately adjacent to the overlapping sequences. LguI cuts outside its recognition site, and cleavage of PCR products occurs within the overlap sequences, liberating a full-length HBV genome that can circularize without the gain or loss of nucleotides.

Rolling circle amplification. Amplification primers for both plus and minus strands were chosen from well-conserved regions of the HBV genome (Table 1). To protect primers from the strong proofreading activity of the *Phi29* polymerase, the two 3′ bases were phosphorothioate modified. Appropriate amounts of DNA (shown in legends to Fig. 1 to 4) were mixed with the eight primers at a concentration of 0.5 μM each and a 1× *Phi29* buffer in a final volume of 10 μl. The DNA mixture was denatured at 95°C for 3 min and then cooled to room temperature in stages: 50°C for 15 s, 30°C for 15 s, and 20°C for 10 min before it was placed on ice. Sample mixtures were combined with 10 μl of reaction mixture containing: 1× *Phi29* buffer, the eight primers at a concentration of 0.5 μM each, 0.4 mg/ml of bovine serum albumin, 4 mM of dithiothreitol, 2 mM of each dNTP, and 10 U of the *Phi29* DNA polymerase (New England Biolabs, Worcester, MA). Reactions were carried out at 30°C for 18 h and terminated at 65°C for 10 min.

RCA product analysis. First, 2 μl of RCA products was run on an 0.8% agarose gel and stained with ethidium bromide. Second, 4 μl of RCA product was digested in a final volume of 10 μl with 3 U of SpeI (New England Biolabs) that cuts only once in most HBV genomes. Digestion products were analyzed on a 1% agarose gel. Full-length genomes amplified by RCA and then genomic PCR were cloned into pCR2.1-TOPO (Invitrogen, Cergy Pontoise, France) and fully sequenced.

Transfection of HuH7 cells. HuH7 cells were grown in Dulbecco's modified Eagle's medium-F12 supplemented with 10% fetal calf serum and antibiotics. Cells were seeded at a density of 5×10^5 cells per well in six-well plates 24 h before transfection. Full-length HBV genomes were excised from cloning vectors using SpeI or LguI (an isoschizomer of Sap I; Fermentas, Vilnius, Lithuania), depending on how they had been cloned. Total DNAs (HBV genome plus vector) were treated with phenol-chloroform, ethanol precipitated, and resuspended in sterile water at an approximate concentration of 1 μg/μl. Exact concentrations were measured by A_{260}. The DNAs were transfected (2 μg per well for single transfections, 1 μg per well of each DNA for cotransfections) using 2 μl per μg of DNA of TransIT-LT1 (Mirus, Madison, WI) according to the manufacturer's instructions. Medium was replaced on the following day (day 1) with medium containing 0, 10, or 100 μM lamivudine (LAM). Two wells were used for each DNA and LAM concentration. Medium contents were changed each day until day 5, and the spent medium was clarified and stored at 4°C until analysis was carried out. HBsAg levels in medium samples pooled from the different days were quantified using a commercial enzyme-linked immunosorbent analysis, MonoLisa HBsAg Ultra (Bio-Rad, Marnes-la-Coquette, France), and a standard curve generated by using purified HBsAg. To be within the linear range of the assay (A_{450}, <1), samples were diluted with normal human serum. On day 5, core-associated intracellular HBV DNA was isolated as described previously (11), electrophoresed, blotted onto a nylon membrane, and hybridized using a ^{32}P-labeled full-length HBV genomic probe. Hybridization signals were quantified by using a PhosphorImager.

Nucleotide sequence accession numbers. The sequences of the HBV genomes have been deposited in GenBank under accession numbers AM295796 to AM295800 and AM410963.

RESULTS

Adaptation of RCA for the amplification of RC-DNA and cccDNA HBV genomes. After the primer and *phi29* polymerase concentrations were optimized, RCA was performed with different HBV DNA templates, as follows: (i) the partially double-stranded RC-DNA (6); (ii) the RC-DNA after the plus-strand DNA was completed using the endogenous HBV polymerase activity; (iii) the treated RC-DNA, ligated after extraction; and (iv) the liver-derived cccDNA (6). Southern blot analysis (Fig. 1) shows that RCA could efficiently amplify

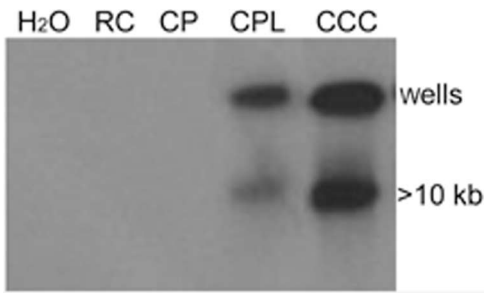

FIG. 1. RCA using different HBV DNA templates. Southern blot hybridization of RCA products. H_2O, negative control; RC, RC-DNA; CP, RC-DNA with plus-strand completed using the endogenous polymerase activity of HBV virions. CPL, CP ligated after DNA extraction. CCC, cccDNA.

only completely circular DNA templates such as HBV cccDNA and the completed/ligated serum HBV DNA. Amplification of serum HBV RC-DNA or HBV RC-DNA that had only been completed but not ligated did not amplify. Also, ligation alone did not permit amplification (not shown). Completion of plus-strand DNA is therefore essential, and it is probably this strand that is ligated. Consequently, in the absence of pretreatment, RCA can discriminate highly between HBV cccDNA and RC-DNA. The pre-S/S region of the genome amplified from serum-derived DNA was directly sequenced, and the sequence was identical to that of the genome originally amplified by conventional techniques (16).

Serial 10-fold dilutions of HBV cccDNA, extracted from the liver tissue of a chronic hepatitis B carrier and quantified by real-time PCR, were used to determine the assay sensitivity. Electrophoretic analysis and ethidium bromide staining show that DNA amplification occurred for all samples tested, including the control reaction mixture without the template added and the sample from an HBV-negative patient (Fig. 2A). Consequently, even though HBV-specific primers were used, nonspecific amplification can occur. This can be explained by self-priming and extension of the primers and by nonspecific amplification that occurs because RCA is carried out at 30°C. However, after Southern blotting and hybridization of the same gel, using a specific HBV oligonucleotide probe, a hybridization signal was observed only with HBV DNA samples and down to the level of 10 copies/reaction (Fig. 2B). Two methods were used to recover unit-length HBV genomes. First, RCA products were digested with SpeI, which cuts only once in the HBV genome. A clear and specific band of linear full-length HBV DNA (verified by hybridization [not shown]) can be seen (Fig. 2C), and exploitable amounts are obtained with the reaction initiated with 10^2 copies of HBV cccDNA. Second, an HBV genomic PCR (7) was performed with the RCA products. A clear ethidium bromide-stained band of the expected size can be obtained from the RCA reaction product originally amplified from 10 copies (Fig. 2D). Thus, the sensitivity of this combination is 10-fold higher than that of the restriction method and is comparable to the sensitivity of real-time PCR. Finally, we have tested the HBV genomic PCR directly with the same serial dilutions of HBV cccDNA without prior amplification by RCA. Analysis shows only a weak band with the sample initially containing 10^3 copies/reaction (Fig. 2E). Consequently, the association between RCA and specific full-length HBV PCR enables a significant increase in sensitivity.

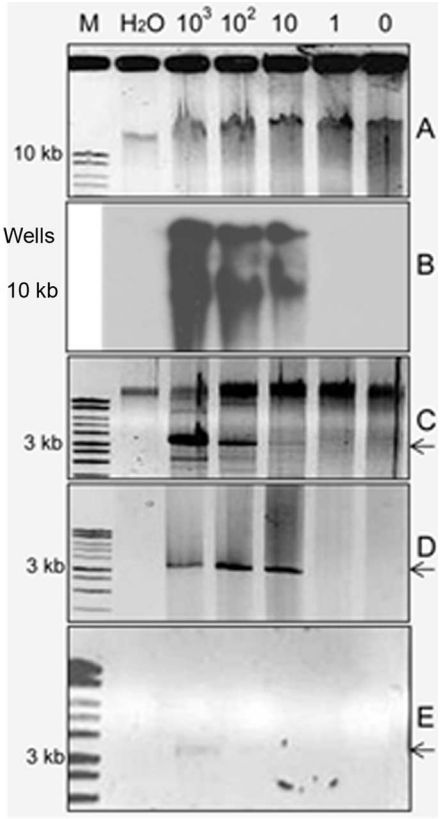

FIG. 2. Amplification of HBV genomes from cccDNA. The number of copies of cccDNA initially present in each RCA reaction is shown at the top. M, molecular weight marker (the sizes of selected bands are shown on the left). H_2O, negative control. 0, negative control (the normal human serum used for the cccDNA dilutions). (A) Ethidium bromide-stained agarose gel of the raw RCA products. (B) Southern blot hybridization of the same gel. The position of the wells that retain some of the RCA products and the position of the high-molecular-weight products (>10 kb) that migrate in the gel are shown. (C) Ethidium bromide-stained agarose gel of the RCA products digested with SpeI. (D) Ethidium bromide-stained agarose gel of the products of a PCR performed with the RCA products. (E) Ethidium bromide-stained agarose gel of the products of a PCR performed directly with cccDNA. In panels C, D, and E, arrows on the right point out full-length (3.2-kb) HBV genomes.

Amplification and molecular characterization of HBV genomes from serial liver biopsy specimens. Three sequential liver biopsy specimens from a patient who had undergone several antiviral treatments were available (17). The first biopsy was performed before the patient entered a dose-finding entecavir trial. The patient was receiving the lowest dose (0.1 mg/kg of body weight/day), and after 6 months of treatment, HBV serum DNA levels had dropped by less than 1 log. A second biopsy was then performed, and the patient was switched to lamivudine but developed resistance to the drug. Line probe assays indicated that the HBV genome was genotype A and contained the mutations rtL180M/rtM204V ("rt" indicates reverse transcriptase domain), associated with lamivudine resistance. Adefovir dipivoxil was added to the regimen, and serum HBV DNA levels gradually declined to an undetectable level, accompanied by the loss of HB e Ag (HBeAg) and HBsAg, followed by seroconversion to anti-HBs 8 months later. The patient did not seroconvert to anti-HBe. The third biopsy was performed at 5 months after seroconversion, and over a year later, the serum HBV DNA level had become undetectable. The levels of cccDNA in the biopsy specimens, estimated by real-time PCR, are shown in Table 2. HBV genomes were amplified from all three biopsy specimens, using RCA, followed by the HBV genomic PCR (Fig. 3) and cloned. A genome (liver biopsy 1, clone 5 [LB1Cl5]) amplified from the first biopsy was fully sequenced. As expected, it was of genotype A and, consistent with the fact that the patient was $HBeAg^+$, contained neither a precore mutation (22) nor core promoter mutations (12). Serum drawn at the same time the biopsy was performed was no longer available, and RCA could not be used to amplify circulating genomes. However, we did

TABLE 2. Real-time PCR quantification (12) of cccDNA[a]

Sample	Mean copies cccDNA/ cell ± SD	Mean copies cccDNA/ RCA reaction ± SD
LB1	1.98 ± 0.04	1,888 ± 180
LB2	1.015 ± 0.035	2,051 ± 86
LB3	0.028 ± 0.01	13.25 ± 5.17

[a] Values are the means ± standard deviations (SD) from two independent quantifications.

have DNA extracted from this serum, and full-length genomes were amplified using the genomic PCR. The PCR product was directly sequenced and was found to be identical to that of the clone amplified from the biopsy. One of the clones amplified from the biopsy (LB1Cl10) displayed a restriction pattern that was different from the other clones. It was fully sequenced and, apart from two single nucleotide substitutions, the only major difference from the first clone was a 129-bp deletion in the PreS1 region (Table 3). It eliminates residues 57 to 99 of the PreS1 protein and most of the S promoter. This genome is therefore probably not viable by itself but can potentially be *trans* complemented by a wild-type genome. One clone (LB2Cl6) amplified from the second biopsy was fully sequenced and was completely identical to LB1Clone5. Mutations that could explain the nonresponse to entecavir were therefore not seen with this clone. Two genomes isolated from the third biopsy (LB3Cl21 and LB3Cl24) were fully sequenced and were identical to that of LB1Clone5, with two important exceptions. These exceptions were that both clones contained the substitutions T667A and A739G that introduce the lamivudine resistance-associated mutations, rtL180M/rtM204V, into the viral polymerase. This shows that the genomes were amplified from the third biopsy, the only one performed after the emergence of lamivudine-resistant mutants. Therefore, more than a year after the disappearance of the serum HBV DNA, viable cccDNA genomes still persisted in the infected liver. However, two types of aberrant genomes were also identified. Some clones showed a restriction enzyme profile consistent with a large deletion. Two clones were fully sequenced, and both possessed an identical 388-bp deletion (positions 2025 to 2412), covering most of the core protein gene. The deletion creates a core-*pol* fusion that will produce a protein containing the spacer, reverse transcriptase, and RNase H domains of the viral polymerase. Both clones possess the two lamivudine resistance substitutions, and apart from these and the deletion, the sequence of one clone (LB3Cl1) is 100% identical to that of LB1Clone5, and the other clone (LB3Cl10) has a single nucleotide substitution. The other type of aberrant genome was found in a clone that contains not only the two lamivudine resistance substitutions plus one other single-nucleotide substitution but also a single-nucleotide insertion (LB3Cl11). The insertion, a G between positions 285 and 286, affects both the polymerase (P) and the surface antigen (S) genes. The insertion frame shifts codon 401 of the P gene into an unused open reading frame, where an additional 65 amino acids can be encoded. The 5′ end of the S gene is fused to the 3′ end of the P gene, encoding a chimera containing 43 residues of HBsAg fused to the last 445 residues of the polymerase.

Functional characterization of the amplified HBV genomes. To test the replication competence and the LAM sensitivity of the clones, LB1Cl5 and LB3Cl24 were transfected into HuH7 cells, and the cultures were treated with different concentrations of the drug. As a control, a wild-type genome of genotype D, 15803, that is known to be replication competent (11) was also transfected. To see whether the two types of aberrant clones can *trans* complement each other, LB3Cl1 and LB3Cl11 were transfected singly and cotransfected. With the exception of LB3Cl11, which has a frameshift mutation in the S gene, all the genomes were capable of synthesizing and secreting considerable quantities of HBsAg (Fig. 4A), including LB3Cl1, which has a deletion in the core/*pol* genes but whose S gene is intact. Intracellular HBsAg levels were also measured (not shown) but represented, at most, 4% of the total HBsAg. HBsAg expression is not affected by LAM treatment, at least on the time scale of this experiment. This is not surprising since LAM affects viral replication and not the synthesis of viral transcripts. In cells transfected with LB1Cl5 and LB3Cl24, in the absence of LAM, the levels of intracellular core-associated HBV DNA were comparable with that of 15803, showing that the genomes are replication competent (Fig. 4B). No core-associated HBV DNA could be detected in cells transfected with the defective clones LB3Cl1 and LB3Cl11, either transfected singly or cotransfected (not shown). In cells transfected with 15803 and LB1Cl5, HBV DNA synthesis is inhibited by LAM treatment in a dose-dependent manner, with 50% inhibition concentrations of 13.9 and 16.3 μM, respectively. At the doses tested, HBV DNA synthesis in cells transfected with LB3Cl24 is completely resistant to LAM treatment.

DISCUSSION

As shown here, RCA reactions can efficiently amplify full-length HBV genomes from circular templates, either cccDNA or RC-DNA that has been completed and ligated. That ligation alone is not sufficient for obtaining amplification suggests that it is completed plus-strand HBV DNA that is initially circularized, despite the fact that plus-strand synthesis is primed by a capped oligoribonucleotide (14). It is probable that this has been eliminated in some RC-DNA molecules. When coupled with a genomic PCR, RCA becomes as sensitive as real-time PCR but with the immense advantage of amplifying full-length genomes rather than small fragments. Using this

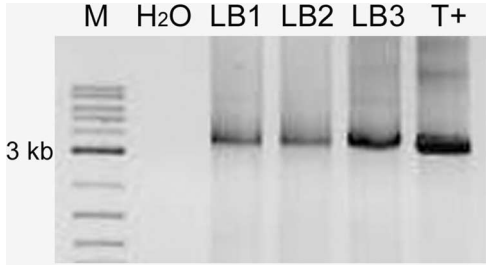

FIG. 3. RCA plus genomic HBV PCR with cccDNA extracted from serial liver biopsy specimens. Ethidium bromide-stained agarose gel of final products of combined RCA and PCR with material from serial liver biopsies. H2O, negative control; LB, liver biopsy; T+, positive control, using a plasmid containing a full-length HBV genome (of genotype C). Both the negative and the positive controls were also subjected to RCA, followed by the genomic HBV PCR.

TABLE 3. Nucleotide differences and resulting lamivudine resistance mutations in the clones described in this study

Clone ID	Substitutions	Deletion (bp)	Insertion	Resistance mutations
LB1Cl5	No	No	No	No
LB1Cl10	G2400A, A2569G	129 (from 3025 to 3153, inclusive)	No	No
LB2Cl6	No	No	No	No
LB3Cl21	T667A, A739G	No	No	rtL180M/M204V
LB3Cl24	T667A, A739G	No	No	rtL180M/M204V
LB3Cl1	T667A, A739G	388 (from 2025 to 2412, inclusive)	No	rtL180M/M204V
LB3Cl10	T667A, A739G, C1279T	388 (from 2025 to 2412, inclusive)	No	rtL180M/M204V
LB3Cl11	T667A, A739G, T1015C	No	One G between 285 and 286	rtL180M/M204V

coupled system, we have successfully amplified virus genomes from several patients whose occult HBV-infected sera could not be amplified using genomic PCR alone. In one case, we could show that the amplified genomes are replication competent (N. Martel and A. Kay, unpublished data). Another major advantage of RCA over PCR is its fidelity (5). Often, genomes amplified using the genomic PCR do not replicate in cell culture even if they are obtained from patients with high levels of HBV replication (8). The sequences of these genomes are often "normal," indicating that apparently anodyne mutations can have important effects on virus replication. If genomes can be amplified using only RCA, then this problem is minimized. If it is necessary to add on a genomic PCR, then this advantage is lost, but it should be possible to further optimize the RCA step and reduce the number of cycles in the genomic PCR. The reliance of RCA on a circular template for efficient amplification is also an advantage. In infected cells, only cccDNA will be efficiently amplified and not integrated HBV sequences. We have used RCA to specifically amplify cccDNA from cells transducted with an HBV/baculovirus vector (15). This ability to directly probe the cccDNA pool will be important for the study of HBV variants, especially the emergence of drug-resistant mutants. The variability of HBV is partly due to the replication strategy of the virus (6). Mutations originally arise in RC-DNA during error-prone reverse transcription and can be stably propagated only by their recruitment into the cccDNA pool. This can happen in two ways: the nucleocapsid containing the mutated RC-DNA can be recycled to the nucleus to maintain the cccDNA pool, or it can be secreted to infect a new hepatocyte, where RC-DNA is converted into cccDNA. In both cases, the mutated RC-DNA genome will be in competition with nonmutated genomes or other genomes newly mutated during the reverse transcription step. The emergence of HBV variants, including drug-resistant mutants, is therefore a long process that can take months or even years. The capacity to directly probe the cccDNA pool can therefore provide significant insights into this problem. In the case of drug-resistant genomes, these have to integrate the cccDNA pool before they can have an impact, and monitoring of this pool can predict future problems. Studies using RCA can investigate the kinetics of the integration of drug-resistant genomes into the cccDNA pool. This implies serial and closely spaced liver biopsies that cannot be done with human subjects but can be done using animal models, duck or woodchuck.

The cccDNA pool is indeed central to the problem of viral persistence. The half-lives of infected hepatocytes and cccDNA are long, and antiviral drug treatments can substantially reduce RC-DNA synthesis but have little effect on intracellular cccDNA levels (19, 23). Prolonged treatments are therefore necessary, and this increases the potential for the emergence of drug-resistant mutants. Even after apparent virus clearance, cccDNA can persist. This has been documented in the woodchuck model (18) and probably explains HBV reactivation (3, 9, 10). In this study, we have shown that a patient who has seroconverted to anti-HBs and has an apparently resolved HBV infection still retains cccDNA in the liver. The results also illustrate the crucial role of the cccDNA pool in the balance between maintaining genetic stability and reacting to selective pressure. Apart from the lamivudine resistance substitutions found in specimens from the third biopsy, the nondefective genomes isolated from the three biopsy specimens over a period of more than 4 years show, at most, a one-nucleotide difference. Conversely, all of the clones isolated from the third biopsy specimen contain the two lamivu-

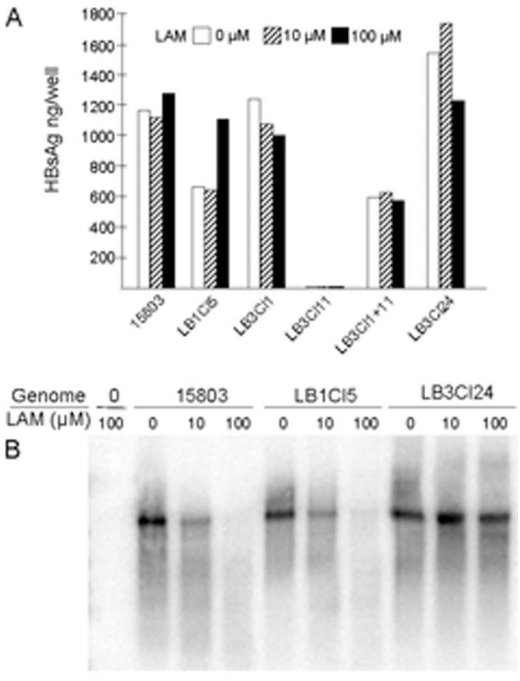

FIG. 4. Transfection of HuH7 cells and lamivudine treatment. (A) A representative transfection experiment measuring the expression and secretion of HBsAg from cells transfected with cloned full-length HBV genomes and treated with lamivudine. (B) Southern blot of intracellular core-associated HBV DNA from cells transfected with cloned full-length HBV genomes and treated with lamivudine. 15803, a wild-type HBV genome of genotype D.

dine resistance mutations, showing that in the face of strong selective pressure, the plasticity of the HBV genome permits the acquisition and propagation of mutations. This shows that resistance mutations can be archived in cccDNA and persist even after seroconversion to anti-HBs. The host immune response is crucial for controlling such a latent infection, and if the patient subsequently becomes immunocompromised, then there is a risk of reactivation. Transfections of genomes amplified by RCA alone (LB1Cl5) or RCA followed by genomic PCR (LB3Cl24) are fully functional, showing that errors were probably not introduced during the amplification process. More importantly, this functional test also permits the confirmation of the phenotypes suggested by the sequences, LAM sensitivity for LB1Cl5 isolated from a biopsy obtained prior to LAM treatment of the patient and LAM resistance for LB3Cl24 isolated from a biopsy obtained not only after LAM-resistant variants had emerged in the patient but also after the patient had seroconverted to anti-HBs. In addition to functional genomes, the accumulation of defective genomes does seem to have occurred after seroconversion and instauration of a "silent" occult HBV infection. They must have been generated during or after the emergence of lamivudine resistance, since they contain the associated mutations. Such defective cccDNA molecules can persist because no viral promoters are affected. However, the retrotranscription of defective pregenomic RNAs into RC-DNA that can then be converted back into cccDNA to maintain persistence of the defective genomes requires *trans* complementation, probably by nondefective genomes. It was possible that the two types of defective genomes could *trans* complement each other (25). However, cotransfection of the two types of defective genomes did not produce detectable levels of replicating HBV DNA. These genomes are therefore probably "dead end" products that will eventually be diluted out by hepatocyte turnover, unless reciprocal recombination between the two types of defective molecules generates a viable genome. It remains to be seen with other patients who have cleared HBV infection whether the persistence of cccDNA together with the accumulation of defective genomes is a general phenomenon.

In summary, RCA will be extremely useful for generating full-length HBV genomes, even from patients with low levels of virus replication, and will permit the direct probing of cccDNA, the central actor in viral persistence and the propagation of mutations. It will also be useful for the phenotypic testing of HBV variants, especially resistance mutations selected by antiviral therapy.

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